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Applicants: De Luca Gilda, Marini Bettolo Rinaldo, Migneco Luisa, Maria

Serial No. 10/531,853

Filed : October 10,2003

Title: Chemical Pharmaceutical Compounds, constituted by derivatives of the taxanes covalently bound to hyaluronic acid or the derivatives thereof “

National Phase of the international Patent Application No.PCT/EP2003/001239 filed on October 10, 20033

**DECLARATION UNDER 37 CFR1.132**

I, Anna Zanellato, being duly sworn depose and say that:

1. I am an Italian citizen residing at: Bovolenta (PD)
2. I am familiar with the English language.
3. I graduated in: BIOLOGY at the University of Padua in the academic year:1987
4. I am author of 19 Scientifical publications.
5. Previous job experiences: From 1987 to 1990 I had worked at the University Department of General Pathology as a researcher, where I had been involved in a study pertaining to smooth muscles cells cultures and in particular to the mechanism of atherosclerosis.

6. Actual job : Since 1990 I have been working at FIDIA FARMACEUTICI S.p.a. in the field of research, involving :

- the analysis of action mechanism of trophic factors,
- studies, utilising neuronal cultures to select new chemical molecules pharmacologically active to prevent different types of neuronal pathologies,
- other studies concerning bovine, rabbit, human , articular chondrocytes cultures on the biomaterials comprising and/or consisting of hyaluronic acid derivatives;

and I, Monica Campisi being duly sworn depose and say that:

7. I am an Italian citizen residing at: Padua
8. I am familiar with the English language.
9. I graduated in : Pharmaceutical Chemistry and Technology at the University of Palermo in the academic year: 2003
10. Ph.D. Student in “Technology of Biologically Active Substances” at the University of Palermo in the academic year: 2007
11. I am author of : 5 scientific publications
12. present Job: Researcher at FIDIA FARMACEUTICI S.p.a., Laboratory of Chemical Research
13. We, Anna Zanellato and Monica Campisi, further declare what follows.

I) correct value of IC50 for taxol on MDA/MB/231 CELLS

The correct datum for taxol IC50 on MDA/MB/231 cells and reported in the table of example 2 in the Specification of the instant Application is not 0.35nM, but 0.35  $\mu$ M as it comes out from a careful reading of figure 2 wherein the ratios of IC50 of taxol vs. the compounds of the invention are reported in the form of a graphic. In fact the correct dimension for the IC50 cannot be nM, but  $\mu$ M.

In fact 0.35  $\mu$ M correspond to 350nm consequently the aforementioned ratio shall be  $350/2.58=135$  coinciding with the value reported in Figure 2.

## II) Analysis of the results reported in the example 2 of the instant Application

In said example 2 the following compound of the invention are tested:

HYTAD1p20 ester derivative of HA covalently bonded to paclitaxel with an esterification degree calculated as w/w of 16%, wherein the average molecular weight of HA is 200,000 Da, corresponding therefore to a substitution degree expressed as % moles of taxol/mole of HA = 9.05%, this meaning therefore that this product contains 9.05 mole of taxol/100 mole of HA (%substitution degree moles of taxol/moles of HA).

HYTAD2p20 ester derivative of HA covalently bonded to paclitaxel with an esterification degree of the carboxyl calculated as w/w of 22%, wherein the average molecular weight of HA is 39000 Da corresponding therefore to a substitution degree expressed as moles of taxol/mole of HA x 100 = 13,4%

HYTAD2p10 ester derivative of HA covalently bonded to paclitaxel with an esterification degree of the carboxyl calculated as w/w of 6.8%, wherein the average molecular weight of HA is 39000 Da, corresponding therefore to a substitution degree expressed as moles of taxol/mole of HA = 3,4%

### **Determination of molar derivatization degree of Hyaluronic Acid (HA) with Paclitaxel, knowing the weight percentage.**

- 1) Calculation of the amount of HA repetitive units where carboxyl groups are derivatized with Taxol-spacer (% R.U. HA-Tax):

$$\%R.U.HA \cdot Tax = \frac{w/w\% \times Mw_{der}}{Mw_{tax}}$$

where:

- w/w % is the weight derivatization degree
- $Mw_{der}$  is the molecular weight of the unit that links taxol-spacer ( $Mw_{R.U.} + Mw_{spacer} + Mw_{taxol}$ )
- $Mw_{tax}$  is the molecular weight of paclitaxel (853.92 g/mol)

- 2) Calculation of the amount of underivatized HA repetitive units, where carboxyl groups are as sodium salt (% R.U. HA Na):

$$\% \text{ R. U. HA Na} = 100 - (\% \text{ R.U. HA-Tax})$$

3) Calculation of the number of **moles** of HA repetitive units derivatized with paclitaxel (mol HA-tax)

$$\text{molHA} \cdot \text{Tax} = \frac{\% \text{R.U.HA} \cdot \text{Tax}}{Mw_{der}}$$

4) Calculation of the number of **moles** of underivatized HA repetitive units (mol HA Na)

$$\text{molHA} \cdot \text{Na} = \frac{\% \text{R.U.HA} \cdot \text{Na}}{Mw_{Na}}$$

where:

-  $Mw_{Na}$  is the molecular weight of underivatized HA repetitive units, where carboxyl groups are as sodium salt (401.38)

5) Calculation of the **molar percentage of Taxol linked to Hyaluronic Acid (HA)**

$$\% \text{molTax} = \frac{\text{molHA} \cdot \text{Tax}}{\text{molHA} \cdot \text{Tax} + \text{molHA} \cdot \text{Na}} \times 100$$

example:

**HYTAD1p20 :**

- w/w % = **16%**

-  $Mw_{der} = (\text{HA Na-Na}) + \text{CH}_2\text{CH}_2\text{CH}_2\text{CO} + \text{Tax} = 1301.41$

-  $Mw_{tax} = 853.92$

% R.U. HA-Tax =  $16 \cdot 1301.41 / 853.92 = \mathbf{24.38}$

% R. U. HA Na =  $100 - 24.38 = \mathbf{75.62}$

$$\text{mol HA-Tax} = 24.38/1301.41 = \mathbf{0.0187}$$

$$\text{mol HA-Na} = 75.62/401.38 = \mathbf{0.188}$$

$$\%molTax = \frac{0.0187}{0.0187 + 0.188} \times 100 = 9.05 \%$$

**HYTAD2p20 :**

$$- w/w \% = \mathbf{22\%}$$

$$- Mw_{der} = (\text{HA Na-Na}) + \text{CH}_2\text{CH}_2\text{CH}_2\text{CO} + \text{Tax} = 1301.41$$

$$- Mw_{tax} = 853.92$$

$$\% \text{ R.U. HA-Tax} = 22 * 1301.41 / 853.92 = \mathbf{33.52}$$

$$\% \text{ R. U. HA Na} = 100 - 33.52 = \mathbf{66.48}$$

$$\text{mol HA-Tax} = 33.52/1301.41 = \mathbf{0.0257}$$

$$\text{mol HA-Na} = 66.48/401.38 = \mathbf{0.165}$$

$$\%molTax = \frac{0.0257}{0.0257 + 0.165} \times 100 = 13.4 \%$$

**HYTAD2p10 :**

$$- w/w \% = \mathbf{6.8\%}$$

$$- Mw_{der} = (\text{HA Na-Na}) + \text{CH}_2\text{CH}_2\text{CH}_2\text{CO} + \text{Tax} = 1301.41$$

$$- Mw_{tax} = 853.92$$

$$\% \text{ R.U. HA-Tax} = 6.8 * 1301.41 / 853.92 = \mathbf{10.36}$$

$$\% \text{ R. U. HA Na} = 100 - 10.36 = \mathbf{89.64}$$

$$\text{mol HA-Tax} = 10.36/1301.41 = \mathbf{0.0079}$$

$$\text{mol HA-Na} = 89.64/401.38 = \mathbf{0.223}$$

$$\%molTax = \frac{0.0079}{0.0079 + 0.223} \times 100 = 3.4 \%$$

If we now convert the values reported in the table of example 2 (corrected as regards the value for IC50 of paclitaxel as underlined in item (I)), in order to determine the IC50 expressed in amounts of taxol equivalents (IC50<sub>taxol equivalents</sub>) contained in the above three tested compounds from the IC50 expressed as nM of tested product (IC50<sub>nM tested compound</sub>) by applying the following mathematical formula

$$\text{IC50}_{\text{taxol equivalents}} = \text{IC50}_{\text{nM tested products}} \times \% \text{substitution degree}_{\text{moles of taxol/mole of HA}}$$

Cells lines	Taxol	HYTAD2p20	HYTAD1p20	HYTAD2p10
MCF/7	3,5 nM	<b>0,11 nM</b> of taxol equivalent	<b>0,0022 nM</b> of taxol equivalent	<b>0,023 nM</b> of taxol equivalent
MDA/MB/231	0,35 $\mu$ M	<b>0,34 nM</b> of taxol equivalent		<b>8,16 nM</b> of taxol equivalent
MDA/MB/468	9,4 nM		<b>0,016 nM</b> of taxol equivalent	
SKBR/3	0,23 nM			<b>0,0047 nM</b> of taxol equivalent

It follows therefore that the compounds of the invention are respectively more effective than taxol on:

- MCF/7 cells respectively:  $3.5/0.11 = \mathbf{31.8 \text{ times}}$   $3.5/0.0022 = \mathbf{1590 \text{ times}}$   $3.5/0.023 = \mathbf{152.1 \text{ times}}$ ,
- MDA/MB/231 respectively  $350\text{nM} / 0.34\text{nM} = \mathbf{1100 \text{ times}}$  ,  $350\text{nM}/8.16 = \mathbf{43 \text{ times}}$
- SKBR/3:  $0.23/ 0.0047 = \mathbf{48.9 \text{ times}}$

III) Comparative analysis between Luo et al compounds cytotoxicity and that of the compounds of the invention

Luo et al. disclose that it is known that HA is over expressed at sites of tumour attachment to the mesentery and provides a matrix that facilitates invasion. Several type of cellular HA receptors

respond to HA as a signal and these include CD44 and RHAMM, the receptor for HA mediated cell motility. (see page 756 lines 21-30).

In view of the above the selectivity for cancerous cells could be markedly enhanced and overall dosages may be reduced by coupling antitumour agents to HA which can produce advantages in drug solubilisation, stabilisation, and localisation.

Consequently the aforesaid authors studied the antitumour activity of the conjugates HA-Taxol linked to each other by means of adipic hydrazide (defined in the publication as HA-ADH-taxol or HA-taxol).

Table 2 at page 760 right column reports different bioconjugate HA-ADH-Taxol having taxol loadings ranging from 1.2% to 15 % by using different ADH loadings (9-18 and 45%) and this taxol loading being a molar ratio as it is the corresponding starting ADH loading, which was calculated by integration of the ADH methylene signals (see page 760 right column lines 24-30).

From the same table it results that, by increasing the taxol loadings the solubility diminishes in fact the HA-taxol with a taxol loading of 14.9%, is partially soluble in water whereas that containing 15% is completely water insoluble.

The cytotoxicity of these HA-taxol compounds was determined and the results thus obtained are represented in table 3 wherein it comes out that for the least modified HA (9% ADH modification) higher toxicity was observed as taxol loading increased, however the cytotoxicity of the highly modified HA (45%) actually decreased at the highest taxol loading (15%) (see page 762 right column line 4 from the bottom-page 762 left column line 3), since high loadings of taxol decreased the solubility of HA taxol-conjugate, masked the HA receptor recognition causing the aggregation of the polymeric conjugate, thus limiting the toxicity of the conjugate relative to the free drug. (see page 762 lines 3-7).

For the above reasons although the HA-Taxol with the highest cytotoxicity is the product 7 having a taxol loading of 14.9%, however this one being partially water soluble, for the subsequent study addressed to compare the cytotoxicity of the conjugate HA-taxol with that of free taxol, the conjugate containing 5% of taxol was selected as it is the most effective and contemporaneously water soluble.

Figure 8 summarise in fact the results of this study.

From this diagram reporting in ordinates the cell viability (%) and in abscissae the concentration of taxol equivalent ( $\mu\text{g/ml}$ ), it results that the IC<sub>50</sub> of the HA-taxol containing a taxol loading of 5% is 0.05  $\mu\text{g/ml}$  versus a IC<sub>50</sub> for taxol as such of about 0.11  $\mu\text{g/ml}$ , thus indicating that this conjugate is at most **twice** more effective than taxol as such.

Apart from the standard error range which is very wide with the consequence that the data reported in this graphic are not statistically significant as admitted by the same authors, Luo conjugates show a cytotoxicity **comparable** to that of free taxol and therefore the above data are decidedly far from the statistically significant data obtained with the compound of the inventions which are from 30 to 1500 times more cytotoxic and therefore more powerful than taxol as such, and in particular those with a low taxol loading of 3.4% (having a more similar taxol loading to that analysed by Luo (5%)) being **152, 43** and about **49** times more cytotoxic and therefore more effective than free taxol.

4. We, Anna Zanellato and Monica Campisi finally declare that all statements made herein of our own respectively biological and chemical knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that such wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the applications or any patents or re-examination certificate issued thereon.

Date: April 15 2008

*Monica Campisi* *Anna Zanellato*

ANNEX 1 FIRST PAGE

N.L. ALLINGER   M.P. CAVA   D.C. DE JONGH  
C.R. JOHNSON   N.A. LEBEL   C.L. STEVENS

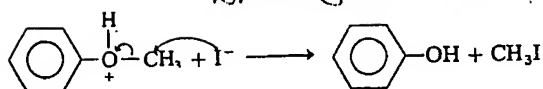
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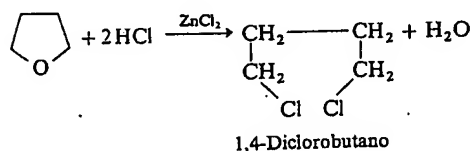
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17  
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ANNE 1 ~~1982~~ Reazioni degli alcoli, dei fenoli e degli eteri 435  
SECOND PAGE



La scissione degli eteri avviene anche in presenza di HBr e HCl. Dato che questi acidi sono meno reattivi dell'HI (per il fatto che  $\text{Cl}^-$  e  $\text{Br}^-$  sono dei nucleofili più deboli di  $\text{I}^-$ ) sono necessarie alte concentrazioni ed elevate temperature perché la reazione sia completa. L'etere ciclico *tetraidrofurano* viene scisso dall'acido cloridrico in presenza di cloruro di zinco per dare l'1,4-diclorobutano, un composto molto importante per la fabbricazione del nylon.



## 18.9. Reazioni degli epossidi

Mentre gli eteri sono molto poco reattivi, gli epossidi (chiamati anche *ossirani* o *ossidi di alchene*, sezione 15.10), sono molto differenti nelle loro proprietà chimiche. Essi reagiscono

Tabella 18-1. Reazioni di apertura dell'anello dell'ossido di etilene

1.  $\text{H}_2\text{C}-\text{CH}_2 + \text{H}_2\text{O} \xrightarrow{\text{H}^+} \begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ | \quad | \\ \text{OH} \quad \text{OH} \end{array}$
2.  $\text{H}_2\text{C}-\text{CH}_2 + \text{CH}_3\text{OH} \xrightarrow{\text{H}^+} \begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ | \quad | \\ \text{OH} \quad \text{OCH}_3 \end{array}$
3.  $\text{H}_2\text{C}-\text{CH}_2 + \text{CH}_3\text{OCH}_2\text{CH}_2-\text{OH} \xrightarrow{\text{H}^+} \text{CH}_3\text{OCH}_2\text{CH}_2-\text{O}-\text{CH}_2\text{CH}_2-\text{OH}$
4.  $\text{H}_2\text{C}-\text{CH}_2 + n\text{H}_2\text{C}-\text{CH}_2 \xrightarrow[\text{H}_2\text{O}]{\text{H}^+, \text{tracce}} \text{HOCH}_2\text{CH}_2 + \text{OCH}_2\text{CH}_2 + n\text{OCH}_2\text{CH}_2\text{OH}$   
Glicole polietilenico
5.  $\text{H}_2\text{C}-\text{CH}_2 + \text{C}_6\text{H}_5\text{O}^- \longrightarrow \text{C}_6\text{H}_5\text{OCH}_2\text{CH}_2\text{O}^- \xrightarrow{\text{H}^+} \text{C}_6\text{H}_5\text{OCH}_2\text{CH}_2\text{OH}$
6.  $\text{H}_2\text{C}-\text{CH}_2 + \text{CH}_3\text{MgBr} \longrightarrow \text{CH}_3-\text{CH}_2\text{CH}_2-\text{OMgBr} \xrightarrow{\text{H}_2\text{O}} \text{CH}_3\text{CH}_2\text{CH}_2\text{OH} + \text{Mg(OH)Br}$
7.  $\text{H}_2\text{C}-\text{CH}_2 + \text{NH}_3 \longrightarrow \begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ | \quad | \\ \text{NH}_2 \quad \text{OH} \end{array} \quad [+ \text{NH}(\text{CH}_2\text{CH}_2\text{OH})_2 \text{ e } \text{N}(\text{CH}_2\text{CH}_2\text{OH})_3]$
8.  $\text{H}_2\text{C}-\text{CH}_2 + \text{CH}_3\text{SH} \xrightarrow{\text{H}^+} \begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ | \quad | \\ \text{CH}_3\text{S} \quad \text{OH} \end{array}$
9.  $\text{H}_2\text{C}-\text{CH}_2 + 2\text{HI} \longrightarrow \begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ | \quad | \\ \text{I} \quad \text{I} \end{array} + \text{H}_2\text{O}$
10.  $\text{H}_2\text{C}-\text{CH}_2 + \text{HCl} \longrightarrow \begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ | \quad | \\ \text{OH} \quad \text{Cl} \end{array}$
11.  $\text{H}_2\text{C}-\text{CH}_2 + \text{HBr} \longrightarrow \text{CH}_3-\text{CH}_2\text{Br}$

ANNEX 2

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Volume 42, Issue 31, 30 July 2001, Pages 5227-5229

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## Preparation of carbamates from amines and alcohols under mild conditions

David D'Addona<sup>a</sup> and Christian G Bochet<sup>a,\*</sup>

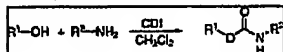
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### Abstract

The practical and mild preparation of carbamates in a two-component system is described by the smooth reaction of an alcohol with 1,1'-carbonyldiimidazole, followed by subsequent trapping with an amine represents a safe replacement for phosgene derivatives.

The efficient and mild coupling of alcohols and amines with 1,1'-carbonyldiimidazole to form carbamates is described. Yields are consistently higher than 83%, and the simple procedure does not require additional purification.



**Keywords:** carbamates; phosgene; two-component system; protecting groups

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Activation of sepharose with *N,N'*-disuccinimidyl carbonate

Journal Applied Biochemistry and  
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Publisher Humana Press Inc.  
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Issue Volume 11, Number 3 / June, 1985  
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DOI 10.1007/BF02798475  
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1. Leckband, Deborah (1991)  
An approach for the stable  
immobilization of proteins.  
*Biotechnology and  
Bioengineering* 37(3)  
[CrossRef]
2. Zeleke, Jessica M. (2006)  
Enantiomer separation of  
amino acids in  
immunoaffinity micro LC-MS.  
*Chirality* 18(7)  
[CrossRef]
3. Zeleke, Tigabu K. (2005)  
Stereoselective antibodies to  
free  $\alpha$ -hydroxy acids. *Journal  
of Molecular Recognition* 18  
(4)  
[CrossRef]
4. Hofstetter, Helke (2005)  
New developments in the  
production and use of  
stereoselective antibodies.  
*Chirality* 17(s1)  
[CrossRef]
5. Bayer, Edward A. (1990)  
Avidin column as a highly  
efficient and stable  
alternative for  
immobilization of ligands for  
affinity chromatography.  
*Journal of Molecular  
Recognition* 3(3)  
[CrossRef]
6. Franco, Elliott J. (2006) A  
comparative evaluation of  
random and site-specific  
immobilization techniques  
for the preparation of  
antibody-based chiral  
stationary phases. *Journal of  
Separation Science* 29(10)  
[CrossRef]

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Israel

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**Abstract** A method is described for the activation of Sepharose  
with *N,N'*-disuccinimidyl carbonate. The activated carbonate reacts  
smoothly with amine-containing ligands yielding stable  
carbamate derivatives.

**Index Entries** Activation, of Sepharose with *N,N'*-disuccinimidyl  
carbonate - Sepharose, activation with *N,N'*-disuccinimidyl  
carbonate - *N,N'*-disuccinimidyl carbonate, activation of  
Sepharose with

References secured to subscribers.